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TITLE: Mitochondrial Based Treatments that Prevent Posttraumatic Osteoarthritis in a Translational Large Animal Intraarticular Fracture Survival Model

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14. ABSTRACT Posttraumatic osteoarthritis is a debilitating consequence of intraarticular fractures. Patient outcomes after intraarticular fractures have not improved significantly in spite of improved surgical techniques. Therefore addressing the acute mechanical damage sustained by cartilage, a new treatment paradigm, offers a new approach to improve patient outcomes after such injuries. Military personnel are particularly disabled by intraarticular fractures. In this research, novel interventions directed at injury-related mitochondrial dysfunction and chondrocyte energy dysfunction are being tested in a small animal and a large animal translational model. These interventions have been successful in tissue level experiments. The interventions scavenge injury related excessive mitochondrial oxidants or prevent their formation, in addition to stimulating glycolytic ATP production. The treatments are designed to prevent chondrocyte death and improve chondrocyte function after an injury. The interventions will be used in an established rabbit model and subsequently in a porcine model of posttraumatic osteoarthritis.					
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Introduction

Experimental Overview

The purpose of this research is to investigate a novel therapeutic approach to prevent PTOA by treating mitochondrial dysfunction in chondrocytes resulting from a severe intraarticular injury. We have shown that scavenging excessive injury-related mitochondrial oxidants, or preventing their excessive formation after a severe impact injury to cartilage in a tissue-level model prevented chondrocyte death in a bovine explanted tibial cartilage^{1,2}. Subsequently, we demonstrated that physiologic levels of mitochondrial oxidants, generated in tissue samples subjected to normal loads, were important promoters of chondrocyte glycolytic ATP synthesis³. These paradoxical observations highlight the physiologic relevance that mitochondrial oxidants have in cartilage. Excessive oxidant concentrations cause chondrocyte death, while physiologic levels of oxidants promote necessary intracellular energy production. These findings form the foundation for the treatment strategies employed in this investigation.

In this set of experiments, we have extrapolated our tissue-level observations into a living organ-level line of experimentation. We are subjecting living joints to severe intraarticular injuries and subsequently modulating oxidant levels and energy production to prevent chondrocyte death and dysfunction in an attempt to prevent posttraumatic osteoarthritis (PTOA). The experimental strategy is to scavenge or prevent mitochondrial oxidant production and rescue glycolytic ATP production with metabolic intermediates. Oxidant scavenging will be done with N-Acetylcysteine (NAC), a known oxidant scavenger that was effective in our bovine tissue experiments. Oxidant production will be blocked by two different methods. In one experiment, we will block oxidant production by preventing electron flow through complex I in the mitochondria with amobarbital. In another experiment, oxidant production is blocked by mechanically uncoupling the mitochondria from the cytoskeleton by cytoskeletal dissolution prior to impact using Nocodazole. We have previously demonstrated that cytoskeletal dissolution significantly reduced mitochondrial oxidants after an impact injury⁴.

Reducing intracellular oxidants will likely reduce intracellular ATP levels by downregulating glycolysis. Therefore, we will augment glycolysis with by addition of substrate intermediates including NAD⁺, glutamine, and ARA 290.

Individual (oxidant concentration manipulation or glycolytic enhancement) and combination (oxidant manipulation with glycolytic enhancement) treatments will be investigated and optimized in a small animal living model. Optimized dosing combinations will then be investigated in an established translational model of posttraumatic osteoarthritis PTOA using a porcine hock fracture model developed at our institution.

Summary of Progress

To date, we have made substantial progress in small animal experimentation. We have performed a series of short term experiments using our rabbit model of PTOA and demonstrated that oxidant scavenging with NAC decreased chondrocyte death in the acute period (7 days) after an impact injury. In addition, injured specimens receiving NAC did have slight decreases in ATP and more significant decreases in proteoglycan concentrations compared to untreated specimens.

We have embarked on short term experiments in the rabbit model investigating both oxidant formation prevention (amobarbital) and glycolysis enhancement (NAD⁺ and glutamine). In addition, we have embarked on medium-term (6 week) and long-term (6 month) experiments with NAC.

Control experiments using our large animal model are progressing. To date ten Yucatan pigs have been successfully fractured using our intraarticular fracture model and sacrificed at 12 weeks.

Eight more pigs have been successfully fractured and are currently recovered in a pen awaiting sacrifice. Our fracture techniques are producing consistent intraarticular fractures.

Body

The three Specific Aims, the associated Statement of Work, and progress to date are outlined below:

Specific Aim 1. Measure changes in chondrocyte ATP production, oxidant production, biosynthetic activity, viability, and PTOA in a survival rabbit model of cartilage injury treated with oxidant scavenging or electron transport complex I inhibition. Subsequently, determine the therapeutic effect of adjuvant glycolytic enhancement.

Task 1: Surgical injury to the rabbit medial femoral condyle (months 1-18)

1. Experimental Groups (sacrifice at either 7 days, 42 days, or 6 months)
 - a. Amobarbital
 - b. NAC
 - c. NAD
 - d. Amobarbital with NAD
 - e. NAC with NAD
2. Control groups
 - a. Impact controls (injury with no treatment)
 - b. Sham control (surgery no injury)
 - c. Normal

Task 2: Confocal, Biochemical, and Histologic Analysis (months 1-21)

1. Confocal Imaging (live cell imaging; oxidant production)
2. Biochemical Analysis (ATP content; proline incorporation; proteoglycan content)
3. Histologic Analysis (Safranin O for Mankin scores; immunohistology for MMP3 and MMP13)

Work to Date Specific Aim 1

Short term experiments have been performed subjecting the rabbit knees to a posterior femoral condyle impact with medial meniscus destabilization. Initially we sacrificed rabbits at either 3 days or 7 days. These preliminary experiments dictated that for short term experiments, sacrifice at 7 days would best show us any treatment effects.

Subsequently, four groups of 10 rabbits have been subjected to the medial femoral condyle injury and have either had no treatment, treatment with the carrier but without NAC, treatment with NAC immediately after impact, or treatment with NAC at three timepoints after impact (immediate, 6 hours, 24 hours). Rabbits were sacrificed at day 7 post-injury. All groups have had confocal imaging after staining specimens with Calcein-AM at the time of sacrifice. Subsequently, specimens have had measurements of tissue content of proteoglycans, proline incorporation, and ATP concentration.

Cell counts demonstrated a trend that NAC increased chondrocyte viability if rabbits received multiple doses after impact. In contrast, a single post-impact dose did not improve chondrocyte viability (Figure 1). None of the findings were statistically significant. There was also wide variability in viability as demonstrated by the large standard deviations. Our most credible conclusion is that at

the current dosing, it is unknown if the NAC improves viability. It is possible but further testing is needed.

Biochemical tests have shown that injury caused decreases in ATP concentrations. Interestingly, treatment with NAC did not improve ATP production compared to untreated specimens (Figure 2). This finding was anticipated. We hypothesize that oxidant scavenging properties of NAC appeared to be effective in preventing cell death, but significantly reduce oxidant levels to a concentration that glycolytic ATP production was affected. This potentially opens a window of therapeutic opportunity for glycolytic enhancing treatments in combination with antioxidants.

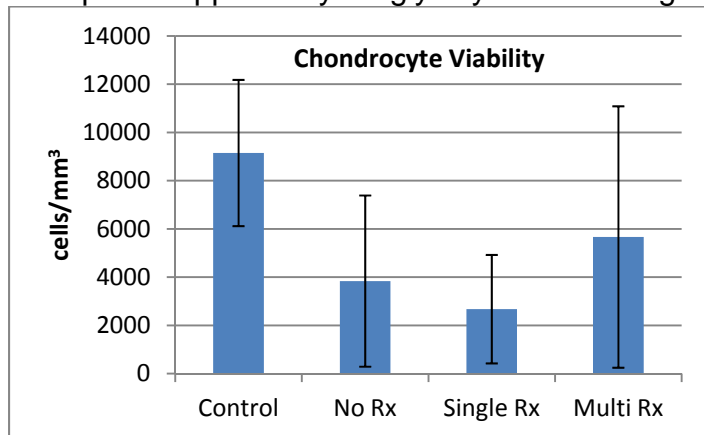


Figure 1. Viable chondrocyte counts in untreated and treated specimens seven days post-impact. Specimens receiving three doses of NAC had greater viability than specimens receiving only one NAC treatment or no treatment.

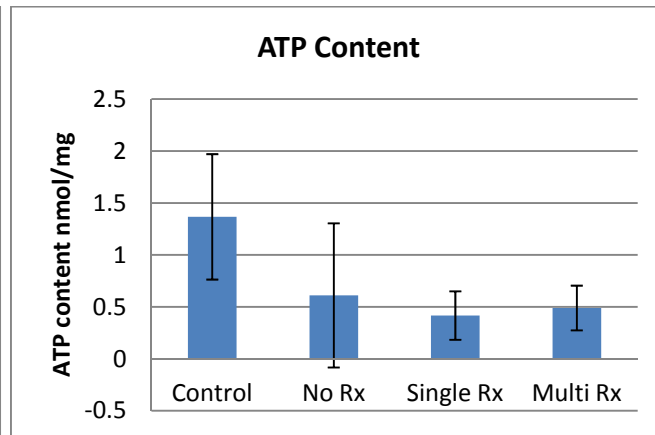


Figure 2. Tissue ATP concentrations seven days post-injury. Oxidant scavenging did not increase ATP concentrations.

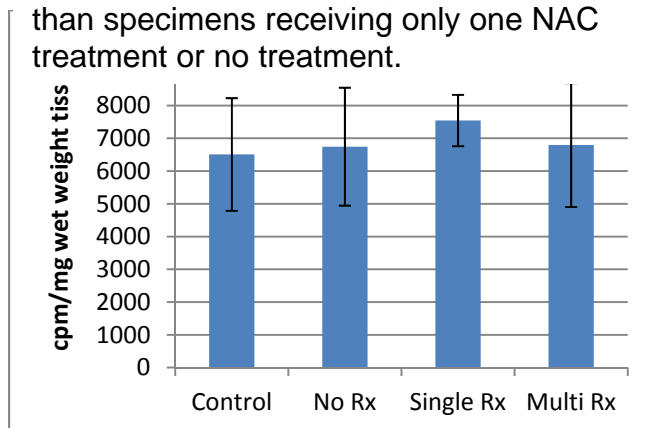


Figure 3. Proline incorporation was not affected by NAC treatment.

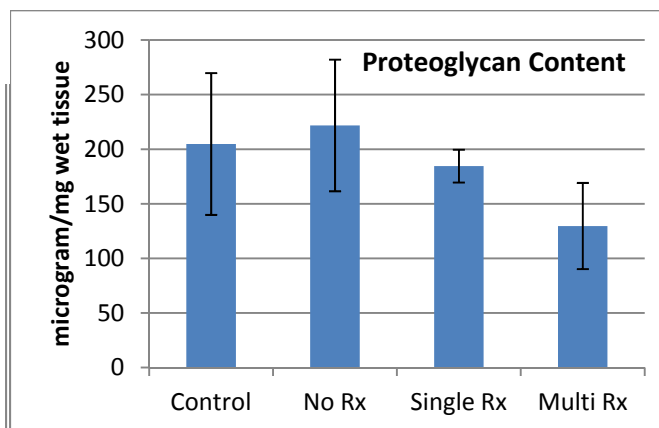


Figure 4. Proteoglycan content was

decreased in NAC treated specimens cted by injury or NAC treatment (Figure 3). In contrast, proteoglycan concentrations were reduced in treated specimens compared to untreated specimens (Figure 4). These findings are consistent with tissue-level investigations that are ongoing in our laboratory. Changes in proline incorporation due to impact injury have negligible in the acute (up to 14 days post-impact) period in bovine cartilage subjected to similar impacts. In contrast, bovine experiments have shown that at both 7 and 14 days post-impact that proteoglycan concentrations increase in response to injury for reasons that are not delineated. Interestingly, proteoglycan concentrations in the current experiment are reduced in treated specimens. Previous studies have shown that proteoglycan synthesis parallels ATP availability ⁵.

Therefore it is possible that in the current study the NAC-related reduction in oxidants and subsequently ATP levels may explain the decrease in proteoglycan content we measured. Again, this opens potential therapeutic opportunities associated with glycolytic enhancing interventions.

Originally, we had planned on three days duration for the short term experiment sacrifice timing. Based on experimentation, this has been modified to seven days. A total of 56 rabbits have been tested to date through completion of the experiment (surgery to complete data collection). Based on our initial findings, there is potential that the NAC doses that were effective in our tissue level experiments are not optimal doses in this animal model. Therefore, we have embarked on a second set of short term experiments in which the concentration and the dosing schedule will be modulated using NAC. In addition, we are performing more tissue-level (bovine tibial plateau cartilage model) dosing experiments using Amobarbital, and our glycolytic enhancing interventions including NAD⁺, ARA 290, and glutamine (these tissue-level experiments are being internally funded). Once tissue-level dosing optimization is complete, we will continue the short-term (7 day) and the longer term (6 week and 6 month) small animal experiments.

Specific Aim 2: Measure changes in chondrocyte ATP production, chondrocyte ROS production, chondrocyte biosynthetic activity, chondrocyte viability, and PTOA in a survival rabbit model of cartilage injury treated with compounds that dissolve filamentous actin and microtubulin. Subsequently determine the therapeutic effects of adjuvant glycolysis-enhancing substrate with cytoskeletal dissolution agents.

Task 1: Surgical injury to the rabbit medial femoral condyle (months 1-18)

1. Experimental Groups
 - a. Cytochalasin B
 - b. Nocodazole
 - c. Cytochalasin B with NAD
 - d. Nocodazole with NAD
2. Control Groups (same as Specific Aim 1)

Task 2: Same as Specific Aim 1

Work to Date Specific Aim 2

None of the Specific Aim 2 experiments have started yet. We are embarking on short term experiments using the Cytochalasin B and Nocodazole.

Specific Aim 3: Determine the efficacy of treatments that prevent ROS overproduction, scavenge ROS, or dissolve the cytoskeleton in mitochondria on preventing PTOA in a large animal IAF survival model.

Task 1: Surgical creation of a physiologic realistic intraarticular fracture in the Yucatan minipig. Once treatments are optimized in the small animal model, we plan on investigating the optimized treatments in the minipig model.

1. Experimental Groups (months 24 – 40): we plan on investigating four different treatment combinations.

2. Control Groups (months 1 – 15)

- a. Injured controls
- b. Uninjured Controls

Work to Date on Specific Aim 3

We have made significant progress on developing the large animal intraarticular fracture model. A total of 18 minipigs have had an intraarticular fracture of their hock joint and have been surgically stabilized in either an anatomic position with rigid fixation, an anatomic position with semi-rigid fixation, or in a malreduced position. This work has been partially funded with this grant, and is also being funded by another DOD Grant (Yuki Tochigi Principal Investigator; Todd McKinley Co-Principal Investigator). Ten of the 18 pigs have made it to sacrifice. Figures 5, 6, and 7 demonstrate representative gross, xray, and histologic findings.

In summary, rigid anatomic fixation had mild to minimal gross PTOA changes 12 weeks after injury with mild histologic changes. Semi-rigid anatomic fixation had significantly increased gross changes and histologic progression of PTOA. Finally, malreduction had more advanced gross PTOA changes with fibrous tissue consistently filling the malreduced defect. In addition, we had 3 nonunions in the stepoff group (see Figure 6, bottom row). Based on these findings, our current strategy will be to investigate the use of the rigid anatomically reduced and the semi-rigid anatomically reduced techniques to serve as injured controls. We are going to operate on six specimens with all reduced anatomically with either rigid (n = 3) or semi-rigid (n = 3) fixation and sacrifice the animals at 6 months. We anticipate that the semi-rigid anatomic specimens will be the best model for PTOA, however final decisions on injured control animals will be made at that time.

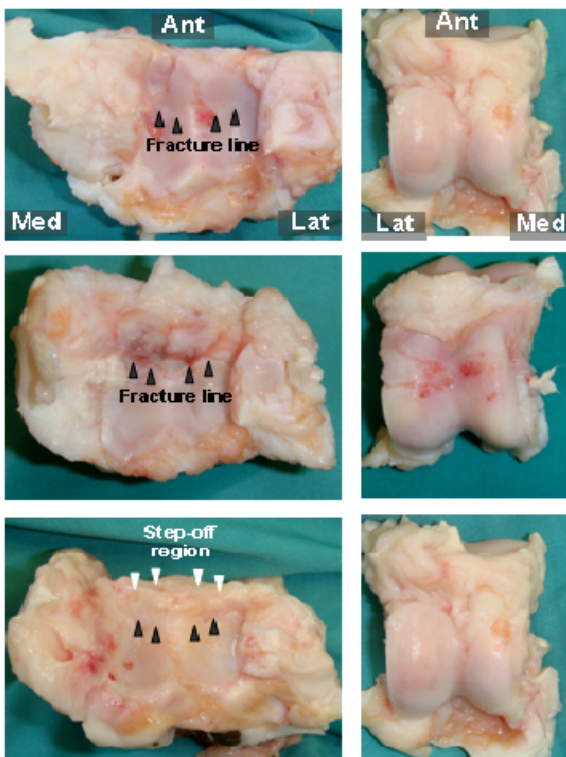


Figure 5. Gross anatomic findings twelve weeks after an intraarticular fracture of the hock joint. The disarticulated tibial surfaces (left) and the corresponding talar surfaces (right) are shown after anatomic reduction and rigid fixation (top), anatomic reduction with semi-rigid fixation (middle); and after a 2.0 mm stepoff with rigid fixation. The fracture healed the reduced cases. Very little degenerative change is evident in the top specimen. In contrast, there are obvious erosive changes both on the tibial and talar surfaces after anatomic reduction with semi-rigid fixation (middle specimen). In the stepoff specimen (bottom), while it appears less erosive than the middle specimen, the entire anterior one-third of the distal tibia is void of cartilage and filled with fibrous tissue (white arrows at stepoff region).



Figure 6. Serial xrays from the same specimens in Figure 5 collected just prior to surgery and then at 2 weeks, 8 weeks, and 12 weeks (time of sacrifice) postoperatively. Degenerative changes are evident in the semi-rigid anatomic group (middle series white arrow). The stepoff is clearly visible in the lower series of xrays with a nonunion at 12 weeks (yellow arrow).

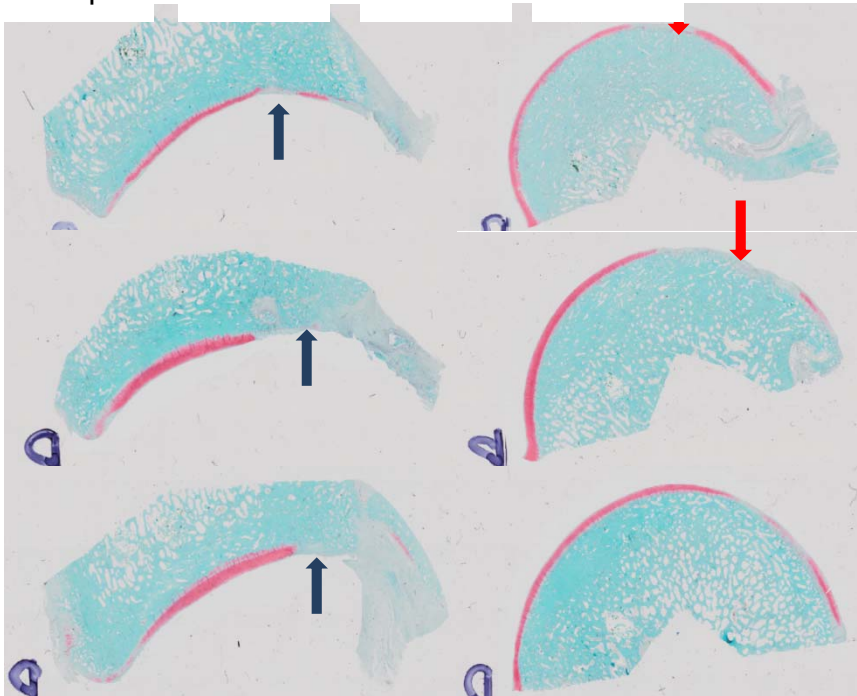


Figure 7. Histologic findings at 12 weeks post-op demonstrate that rigid anatomic fixation (top row) minimized tibial sided cartilage erosion (dark arrows) compared to semi-rigid anatomic fixation (middle row) and the stepoff reduction (bottom row) specimens. Talar erosion was also reduced in rigid anatomic fixation compared to semi-rigid fixation (red arrows). Little talar erosion was evident in the stepoff group.

Key Research Accomplishments

1. Small animal model is functioning as anticipated. The rabbits are tolerating the surgery and remain active in the immediate postoperative period. The model is yielding the anticipated data sets demonstrating that it is a feasible model to conduct the investigation.
2. Significant progress has been achieved in implementing the large animal model. Again, the pigs have uniformly survived and become active after the operation. The model is producing a hock joint with demonstrable PTOA in a highly reproducible manner with consistent coronal fractures of the anterior distal tibia.
3. There is a trend, but no definitive evidence, that multiple dosing of oxidant scavenging (NAC) improves chondrocyte viability in a short term experiment. In contrast, it appears that oxidant scavenging does not improve ATP levels and decreases proteoglycan content in short term experiments.
4. All assays and imaging techniques are functioning normally.

Reportable Outcomes. There are no reportable outcomes at this time.

Conclusions

1. Oxidant scavenging has potential to improve chondrocyte viability after an intraarticular impact injury. Further testing to determine optimal dosing is necessary and ongoing.
2. Oxidant scavenging after an intraarticular impact injury reduces cartilage ATP concentration and proteoglycan concentration. In contrast, there is no effect on protein synthesis as determined by proline incorporation.
3. A porcine intraarticular fracture model consistently results in a coronally-directed intraarticular fracture of the hock joint. Degenerative changes are directly affected by the reduction of the articular surface and by the stiffness of the internal fixation construct.

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